

Aus dem Max-Planck-Institut für Biochemie, München

Direktor: Prof. Dr. Adolf Butenandt

**Untersuchungen zur Reinigung und Anreicherung von
Bakteriophagen und doppelsträngiger, ringförmiger
Desoxyribonukleinsäure**

(Dargestellt am Beispiel des Bakteriophagen ØX 174
und dessen replikativer Form)

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LYSIS INHIBITION OF Φ X174-, M12-, AND Q β -INFECTED *ESCHERICHIA COLI* BACTERIA BY MAGNESIUM IONS

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SUMMARY

1. High concentrations of Mg^{2+} in the culture medium inhibit lysis of Φ X174-infected *Escherichia coli* C bacteria, thus yielding a 5–10-fold higher phage production. 90 % or more of the phages produced under these conditions can be sedimented together with the infected cells. The phages are quantitatively extracted from the bacterial sediment after lysis induced by EDTA.

2. A similar inhibitory effect of Mg^{2+} on the lysis of M12-infected *E. coli* K12 AB and Q β -infected Q13 cells, resulting in a 2–3-fold higher phage yield, is reported.

INTRODUCTION

All small bacteriophages are not easily concentrated and purified from their lysates. For many investigations, however, it is important to have highly purified and concentrated solutions of phages. For this reason, various concentration procedures have been developed. Precipitation of the phages with acids or salts or sedimentation by high-speed centrifugations are time-consuming and often are accompanied by substantial losses. Two-phase distribution procedures using polymers involve less losses but are rather expensive. Several attempts have been made to develop growth conditions which would inhibit lysis of infected bacteria and thus make it possible to extract the phages in highly concentrated solutions from cellular sediments. This has been successful with the help of agents commonly in use to stabilize protoplasts and spheroplasts against osmotic shock. GROMAN AND SUZUKI¹ used spermidine and were able to stabilize Φ X174 and ϕ_2 -infected *Escherichia coli* cells. This stabilizing effect has also been observed in this laboratory by HELLMANN² using cadaverine to stabilize M12-infected *E. coli* bacteria.

This paper reports the use of a more readily obtainable spheroplast stabilizing agent, namely Mg^{2+} . We have investigated the production of Φ X174, M12, and Q β bacteriophages in Mg^{2+} -protected *E. coli* cells in order to develop an improved method of concentrating these phages.

MATERIALS AND METHODS

(a) Bacteriophages and bacterial strains

Φ X174 and M12 bacteriophages came from stock solutions routinely used in this laboratory. Q β bacteriophage was kindly supplied by Dr. Spiegelman (Urbana,

Ill.), *E. coli* C bacteria were used as host for $\Phi X174$, and the *E. coli* strains AB 301 and Q13, kindly given to us by Dr. Gesteland (New York) and Dr. Weissman (Zürich), respectively, were hosts for the RNA-phages M12 and Q β . Standard phage procedures were performed as described by ADAMS³.

(b) *Media and buffers*

MD medium contains per l: 30 g glycerol, 10 mg gelatin, 1.0 g NH_4Cl , 0.3 g $MgSO_4$, 35 mg $CaCl_2$, 1.8 g Tris, 11 g NaCl, 6 g KCl, 5 g glucose, 5 g technical caseamino acids (Difco), 2.5 g yeast extract (Difco), and 950 ml of water. A sterile solution of 1.05 g Na_2HPO_4 and 0.45 g KH_2PO_4 in 10 ml of water was added after autoclaving.

M3 medium contained 17.5 g dehydrated Bacto Antibiotic Medium 3 (Difco) per l of distilled water.

M3G medium was M3 medium supplemented with 4 g glucose per l. In all media to be supplemented with high concentrations of Mg^{2+} , the concentration of either NH_4^+ (M3 medium) or HPO_4^{2-} (MD medium) has to be kept below 0.01 M to prevent precipitation of $MgNH_4PO_4$.

Borate-EDTA solution containing 14.5 g sodium tetraborate and 0.37 g EDTA per l was used for dialysis. Borate-0.05 M EDTA solution for phage extraction contained 14.5 g sodium tetraborate and 18.6 g EDTA per l and was adjusted to pH 9.5.

(c) *Selection of E. coli AB 301 and Q13 bacteria to grow in $MgSO_4$ -containing media*

In contrast to *E. coli* C, both bacteria of strain AB 301 and Q13 have considerably prolonged generation times and, though sensitive to M12 and Q β phages, produce no progeny phages when high concentrations of magnesium salts are added to the medium. For this reason, bacteria able to grow in the presence of magnesium salts were selected by the following procedure: Cultures of AB 301 and Q13 bacteria were diluted to $3 \cdot 10^3$ – $5 \cdot 10^3$ cells per ml, and 0.1 ml was spread on agar plates supplemented with 0.2 M $MgSO_4$. Single colonies from plates which had been incubated at 37° for 20 h were transferred into M3 medium and allowed to grow to $5 \cdot 10^8$ cells per ml. Such cultures were tested for sensitivity to M12 and Q β bacteriophages on standard agar plates.

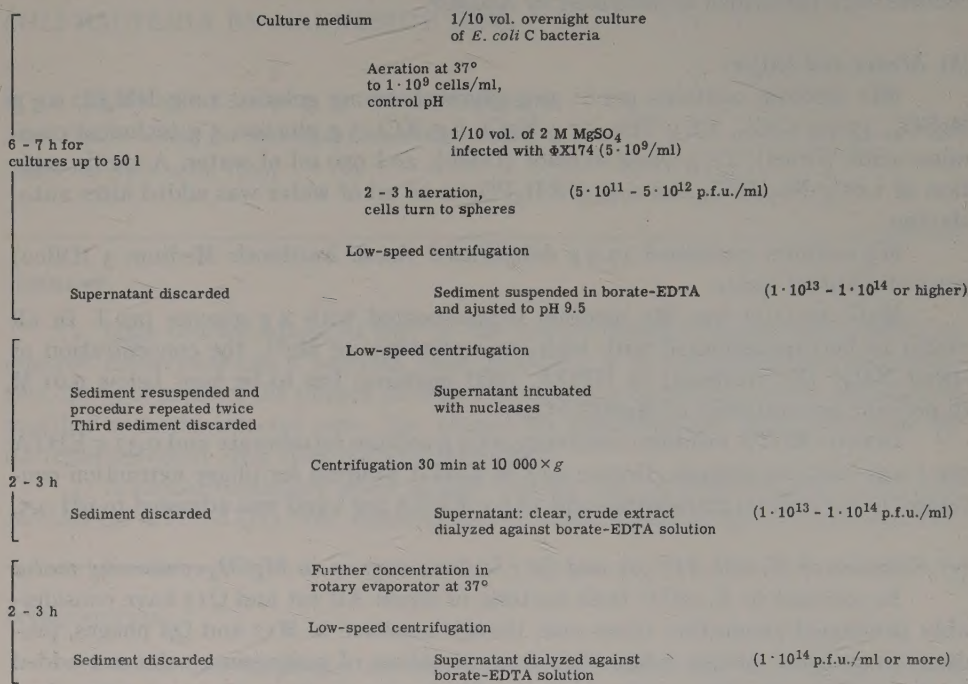
Over 60 % of the single colonies that grew on $MgSO_4$ -containing agar plates was sensitive to M12 and Q β bacteriophages, and almost all of them had normal generation times (30 min or less).

Sensitive bacteria were preserved on slant agar containing 0.2 M $MgSO_4$. No continual passage through Mg^{2+} -containing media was necessary to preserve the ability to grow and produce progeny phage in media containing high concentrations of Mg^{2+} . Overnight cultures were grown in normal M3 medium with aeration at 37°.

(d) *Growth conditions and concentrating procedures for $\Phi X174$ bacteriophages*

Warm MD medium in growth vessels containing 4–12 l was seeded with 1/10 of its volume of overnight culture of *E. coli* C bacteria grown on the same medium and incubated at 37° with vigorous aeration. pH was controlled every 20–30 min and adjusted to pH 7.2 with 1 M NaOH when necessary. When the culture reached $1 \cdot 10^9$ cells per ml, it was adjusted to 0.2 M Mg^{2+} by addition of a sterile 2 M $MgSO_4$ solution. $\Phi X174$ bacteriophages were added to give a concentration of $5 \cdot 10^9$ infec-

tive particles per ml. The culture was aerated for another 2–3 h. Foaming was controlled by silicone antifoam agent (Wacker). The bacteriophages were isolated from such cultures by the following procedure (Scheme 1).



Scheme 1. Preparation and concentration procedure for Φ X174 bacteriophages. All figures given are valid for Φ X174 preparations only. The diagram can, however, be used for the production of RNA phages if the respective figures are inserted according to the description in paragraph (e).

The phage-containing cells were collected by centrifugation. The sediment was suspended in cold borate–0.05 M EDTA solution to give a 50–100-fold concentration of the original culture volume. The suspension was adjusted to pH 9.5 by the addition of 1 M NaOH under vigorous stirring in a blender or vibration homogenizer. Lysis under these conditions caused shearing of the host DNA and thus led to a considerable reduction in the viscosity of the homogenate. Subsequently, most of the cellular debris could be removed by low-speed centrifugation at 3000 × g for 10 min. The sedimented debris was extracted twice with fresh buffer solution, 1/100 and 1/200 of the original culture volume being employed. The supernatants of all three extracts were combined or individually processed as follows. The turbid and viscous solutions were adjusted to 0.05 M MgSO₄ and, after the addition of 10 μg deoxyribonuclease and 10 μg ribonuclease per ml, were incubated at 37° for 30 min. The lysates, no longer viscous, were centrifuged at 10 000 × g for 30 min. The resulting clear and yellowish supernatant was exhaustively dialyzed against borate–EDTA solution. This phage solution was, if desired, further concentrated in a rotary evaporator to 1/10 or 1/20 of the starting volume at 30–37°. Precipitated salts were removed by centrifugation, and the concentrates were immediately dialyzed against two changes of borate EDTA solution.

(e) *Growth conditions and concentrating procedures for M12 and Q β bacteriophages*

Warm M3G medium was inoculated with 1/100 of its volume of fresh overnight culture of AB 301 (Q13) bacteria (selected as described in (c) and aerated at 37°. pH was controlled and adjusted to pH 7.2 with 1 M NaOH, if necessary. The last control was made before the addition of $MgSO_4$. In the presence of high concentrations of Mg^{2+} , no OH^- should be added because $Mg(OH)_2$ is precipitated and remains insoluble. When a titer of $1 \cdot 10^8$ – $1.5 \cdot 10^8$ cells per ml was reached, $MgSO_4$ was added to give a concentration of 0.2 M. 30 min later, at a titer of $2 \cdot 10^8$ – $3 \cdot 10^8$ cells per ml, the bacteria were infected with 20 M12 (Q β) bacteriophages per cell. After another 3–4 h of vigorous aeration, the phage-containing cells were centrifuged at 5000 rev./min for 30 min. The supernatant was discarded, and the pellet was treated in the same way as already described for Φ X174-infected *E. coli* C cells (see paragraph (d) and Scheme 1).

RESULTS

(a) *Production of Φ X174 bacteriophages in Mg^{2+} -protected *E. coli* C bacteria*

Under normal growth conditions, Φ X174-infected *E. coli* C bacteria lyse 35 min after infection. If, however, the medium is adjusted to 0.2 M $MgSO_4$ before infection, lysis does not occur within the first 3–4 h after infection since the outer layers of the cell wall are stabilized by the Mg^{2+} (W. LUETGEB, personal communication).

If Φ X174-infected *E. coli* C bacteria growing under Mg^{2+} protection are observed in the microscope, 20 % of the cells is found to have been converted to spheres 20–30 min after infection. Between 60 and 90 min, all cells are found to be spheres which continue to swell to twice their original size (90 min–150 of infection). The cell concentration is only slightly reduced during this period. These spherical cells obviously continue to produce phage because the final phage titer of Mg^{2+} -protected cultures is 5–10 times that of control lysates, namely $5 \cdot 10^{11}$ – $5 \cdot 10^{12}$ /ml. 90 % or more of phages produced under protective conditions can be sedimented together with the now spherical bacteria. Lysis occurs upon suspending the cells in borate buffer supplemented with EDTA to chelate the Mg^{2+} present in the sediment.

Between 60 and 85 % of the infectious units present in the homogenate remains in a viscous and turbid supernatant after low-speed centrifugation. The two subsequent extractions of the sediment recover another 10–30 %. In all, up to 95 % of the phages can thus be extracted from the bacterial sediment, yielding highly concentrated solutions with typical titers of $1 \cdot 10^{13}$ – $1 \cdot 10^{14}$ /ml or more.

The percentage of phages recovered in the individual extraction steps varies from one experiment to another, but the overall yields are highly reproducible. Higher yields in the first extraction are obtained if the homogenate is allowed to stand in a cold room overnight.

Cellular nucleic acids are degraded by nucleases as described in MATERIALS AND METHODS. At this step a lot of contaminating material can be removed by centrifugation without measurable loss of infectivity from the supernatant.

If necessary, the volume of the crude concentrate may be further reduced in a rotary evaporator with barely detectable loss of infectivity. The final concentrates,

LARGE-SCALE PREPARATIONS OF VIRUSES BY STERIC CHROMATOGRAPHY ON COLUMNS OF CONTROLLED PORE GLASS

 Φ X174, M13, M12, Q β AND T4 BACTERIOPHAGES

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SUMMARY

Several bacteriophages of enterobacteria are quickly and effectively purified from highly concentrated crude extracts using columns of controlled pore glass without detectable loss of infectivity. The nucleic acids of the host cells are degraded by nucleases, and the bacterial debris is sedimented by low-speed centrifugation prior to column chromatography. Highly concentrated solutions of purified bacteriophages are eluted in the exclusion volume of the column.

The preparation of the controlled pore glass columns and principles of operation are described. In the case of the *Escherichia coli* C bacteriophage Φ X174, an exhaustive investigation of purity of glass-column chromatographed phage fractions has been made. For the other bacteriophages, sufficient evidence is presented to conclude that they will be as effectively purified as Φ X174. The chromatographic step successfully removes both bacterial nucleases and the pancreatic nucleases used for degradation of the bacterial nucleic acids.

INTRODUCTION

Because of their simplicity in structure and mode of multiplication, viruses, especially bacteriophages, have been an object of intensive investigation during the last decade both for geneticists and biochemists.

Many problems, such as sequence analysis of coat protein or nucleic acid, the production of antisera, or the isolation of phage-induced enzymes, can be tackled only if large amounts of concentrated solutions of highly purified viruses are easily obtainable.

All current methods of purification are laborious and time-consuming because they require density gradient centrifugations of rather contaminated viral solutions. Therefore, these methods cannot easily be scaled up for large-scale preparations. In this paper a simplified method is presented which allows the purification of large quantities of bacteriophages from crude concentrates in very short times. Chromatography on gels has been an effective method for transferring viruses from concentrated salt solutions (CsCl) into a buffer of desired composition. Yet the pores of such

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gels are too small to allow a separation of viruses from rather high-molecular-weight contaminants. Gels with pores that might be large enough to purify viruses (e.g. agar derivatives), have the disadvantages of all gels in that the pore sizes cannot be determined exactly, and apparently there seems to be no way of producing gels of narrow pore distribution and well-controlled pore size. Furthermore, both pores and column bed are subject to shrinkage or expansion with changes of the ion environment. Gel columns cannot be regenerated or sterilized by heat or strong chemical agents. Finally, column beds of gel tend to pack under the pressure of their own weight and the forces produced by the eluant flow. Flow resistance is therefore rather high, and no high flow rates are attainable. Beset with all these difficulties, we saw no way of developing a quick and easily reproducible purification method with these materials.

One of us has developed a novel type of molecular fractionation using porous glass of controlled pore size¹. The process of pore formation can be controlled in such a way that only pores of a desired size are found², thus making it possible to produce a "tailor made" chromatographic material, which, it was hoped, would be most effective for the problems under investigation. The pore size distribution of such glass can be accurately measured by physical methods³. Columns filled with particles of such glass⁴ have the advantage that, if perfectly packed, the column bed does not shrink or expand under any conditions. They can be autoclaved and can be regenerated with acids or diluted ammonia, should they become contaminated. The glass is inert to practically all substances, including eluants which normally destroy or degrade other column packings. High pressures may be applied without any change of the column bed or pore size; therefore extremely high flow rates are obtained. The narrow pore distribution results in good resolution and high capacity of such columns.

Since controlled pore glass columns possess such favorable properties, this material was selected for the development of a method for large-scale purification of bacteriophages.

MATERIALS AND METHODS

(a) *Preparation of raw bacteriophage-containing concentrates*

Φ X174, M12 and Q β bacteriophages were prepared and concentrated as described by GSCHWENDER AND HOFSCHEIDER⁵.

M13-phage containing concentrates were obtained by precipitation of the phages with $(\text{NH}_4)_2\text{SO}_4$ as described by HOBOM AND BRAUNITZER⁶.

Concentrated T4-phage solutions were kindly supplied by Dr. P. W. K hl of this laboratory.

(b) *Preparation of columns and principles of operation*

Glass grains with pores of controlled size were prepared by one of the authors as previously described, and pore sizes and pore volume were determined as described in the same articles^{1,2}. The glass grains were carefully prepared to give high pore volume, narrow distribution of pore size and channels free from adsorbing silica gel. Column envelopes consisted of glass tubes, 1 m long and 1 cm inner diameter. They were fitted with removable coarse porous plugs at both ends. The lower plug was inserted into the tube which was then clamped in a vertical position and filled with

an aqueous slurry of controlled pore glass under light vibration of the column. The slurry was prepared by adding de-aerated water (boiled and cooled to room temperature) to dry controlled pore glass. If the glass is clean, the pores fill instantaneously with water and no evacuation is needed. The slurry is stirred and left standing for a few seconds until the particles have settled. The excess water is decanted and replaced by fresh de-aerated water. This is repeated 2–3 times, and the slurry is poured into the column. As the particles settle rapidly to the bottom, the clear water at the top is drawn off and more slurry is added. The bed is then compacted by vibrating the envelope until the terminal bed height is reached. This takes approx. 10 min. The top plug is put in place and the column is ready for operation. It is advisable to remove the fines formed during vibration by pumping a few column volumes of water through the column. Fines accumulating before the exit plug cause a temporary increase in flow resistance. This can be reduced by simply reversing the direction of flow 1 or 2 times.

An alternate procedure is to fill the column with dry controlled pore glass and afterwards displace or dissolve the air by pumping water through the column. If the water is deaerated and cold (5°), this takes only a few hours. The easy removal of accidental air inclusions is another advantage of controlled pore glass as column packing. For small columns we prefer the "wet" method, while the "dry" method had advantages for large columns.

The column packings used had been fractionated by sieving to a particle size of either 50–100, 100–200, 120–200 or 80–100 mesh (ASTM). In preliminary tests we found no justification to go to finer fractions. The coarse particles speed up the filling and regeneration procedures and give lowest flow resistance. Furthermore, their resolving power is in no way inferior to that of the finer fractions as has been reported with gels. The complete accessibility of the internal pore system from every point of the grain surface may be the reason for this. The pore volume of the glass averaged 0.7 ml per g.

Precise knowledge of the specific pore volume of the porous packing and its incompressible nature allow one to predict accurately the "exclusion" and the "salt" volume of a column from filling data only. One of us has shown³ that the total free volume of the column (total amount of liquid in column) coincides with the experimentally determined salt volume, measured with benzyl alcohol. Similarly, he found that the void volume of the column (total free volume *minus* pore space) coincides with the exclusion volume as determined with tobacco mosaic virus suspension.

To obtain the necessary filling data for a column filled with a porous glass of known specific pore volume (p), we proceed in the following way. The empty column and all its accessories are weighed before filling (weight a) and after filling with glass particles and water (weight b). In order to obtain the actual dry weight of controlled pore glass in the column, we prepare the slurry in a beaker by weighing into it a known amount of dry controlled pore glass, in excess of the amount which we expect to need for the column. The slurry is prepared in the same beaker, and any slurry not used for the column is collected, dried in an oven and weighed again. The difference gives the dry weight of controlled pore glass actually used for the column (weight c). The total weight of liquid in the column is therefore $b - a - c$. This has to be divided by the specific weight of the liquid (δ) to give the total free volume of the column:

$(b-a-c)/\delta$. Part of this free volume consists of liquid in the pores of the controlled pore glass which have a volume of $c \cdot p$. The void volume is therefore $[(b-a-c)/\delta] - c \cdot p$. We found that these two parameters agree within experimental error with those obtained by actual calibration with tobacco mosaic virus and benzyl alcohol. Calibration has the added advantage of testing the whole chromatographic system. Actual peak positions can further be normalized relative to the above volume parameters, giving position numbers (k) which are independent of column size and column geometry. Further, this allows prediction of the peak position of a substance for any column design. Normalized peak positions (k) can also be used in plots of pore size *versus* k , or molecular weight (within homologous series of substances!) *versus* k , or particle size *versus* k as aids to select pore sizes.

Columns were used over long periods without need for regeneration. Accidental contaminations were removed by injecting 5–10 ml of diluted NH_4OH solution (1 part concentrated commercial ammonia *plus* 5 parts water) which was flushed through the column with water (not buffer!) until the effluent was completely neutral again. We made it a habit to treat columns in this way when they were to be used for different materials or had not been used for some time. Columns which could not be cleaned in this way (*e.g.* after a dirty column had been sterilized in the autoclave) were washed with warm concentrated nitric acid.

In all our experiments the virus was the largest species in the raw concentrate. For this reason, the strategy of selecting the best pore size consisted of starting with a column of small pore size. If the virus appeared in the exclusion volume, we switched to a larger pore size. Our aim was to find a pore size which was just small enough to exclude the virus. This results in maximum separation of virus from smaller contaminants. The use of properly made glass of an appropriate pore size and narrow pore size distribution, together with the possibility of using high flow rates, was the key for economic processing of large batches with relatively small columns. Though, no retention of virus could be found, even if the pores were large enough to admit the virus¹, the principle of starting with a small pore size was always used. This is mandatory if working with very sensitive entities, which may be deactivated or adsorbed by entering the pores. A very sensitive and short-lived enzyme-substrate complex has been successfully purified in another laboratory by using controlled pore glass which had a narrow pore size distribution and a pore size slightly smaller than the complex. This complex is deactivated by chromatography on gels but also on glass grains having a pore size large enough to admit the complex (G. RUHENSTROTH, personal communication).

A peristaltic pump which allowed variation of flow rate between 0.5 and 10 ml/min was used to transfer the elution buffer from a burette into one end of the column. A three-way stopcock allowed alternate sample injection or passage of elution buffer. The exit of the column was connected to an ultraviolet absorption monitor (254 $\text{m}\mu$) and fraction collector. Columns could be operated in the cold room or at room temperature. Owing to the high flow rates, transit time of the sample through the column is so short that refrigeration of the column can be dispensed with. The chromatographic system can be tested by injecting 0.5 ml of 0.1 % tobacco mosaic virus and 1 % benzyl alcohol and eluting with water. The volume of eluant withdrawn from the burette was observed and recorded on the strip chart in parallel with the ultraviolet absorption curve.

All buffers and water used for the columns were boiled and stored under exclusion of air.

RESULTS AND DISCUSSION

Controlled pore glass column chromatography has been used to purify different bacteriophages: the small single-stranded DNA-containing phages Φ X174 and M13; the single-stranded RNA-containing phages M12 and Q β ; and T4, as representative of the large double-stranded DNA-containing phages. All these phage particles have passed through controlled pore glass columns without measurable loss of infectivity.

Concentrated raw extracts containing bacteriophages were prepared by the procedures summarized under MATERIALS AND METHODS. If the extracts contained nucleic acids of the host cells, these were degraded by nucleases and bacterial debris

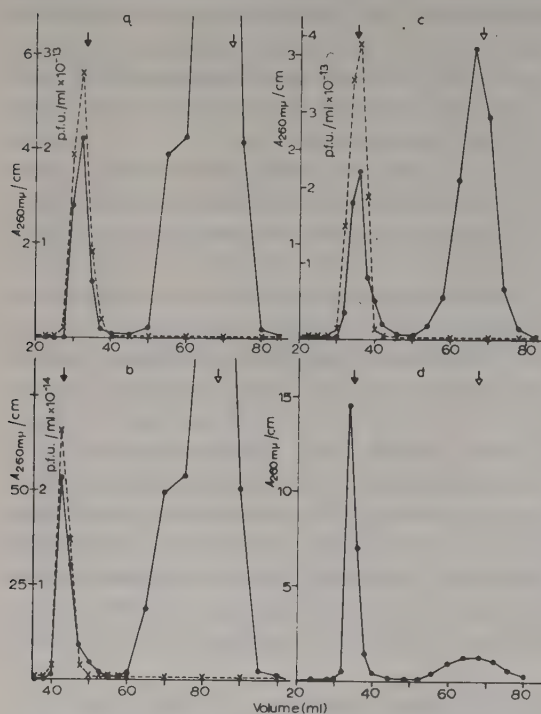


Fig. 1. Elution profiles of phage concentrates purified on controlled pore glass columns. The phage concentrates and columns were prepared as described in MATERIALS AND METHODS. Flow of eluant was 2 ml/min, fractions were collected every 1 or 2 min. ●—●, absorbance at 260 mμ; ×---×, infectivity (p.f.u. = plaque forming units). Arrows mark exclusion (closed arrows) and salt (open arrows) volumes of the columns as determined by calibration with tobacco mosaic virus and benzyl alcohol (see MATERIALS AND METHODS). (a) 3 ml Φ X174 phage concentrate, containing $3.5 \cdot 10^{13}$ infective particles per ml were purified on a controlled pore glass column of 280-Å pore size. (b) 2.5 ml M12 concentrate containing $2.5 \cdot 10^{14}$ infective particles per ml were chromatographed on a controlled pore glass column of 240-Å pore size. Q β phage concentrates gave the same elution pattern on this column. (c) 4 ml M13 concentrate containing $6 \cdot 10^{13}$ infective particles per ml were purified on a controlled pore glass column of 2250-Å pore size. (d) T4 concentrate was chromatographed on a 2250-Å controlled pore glass column. (Infectivity was tested in the pooled peak fractions only.)

was sedimented by centrifugation (30 min at $10\,000\times g$). Dialysis procedures could be dispensed with because in controlled pore glass chromatography the phage particles are eluted in the exclusion volume and thus transferred into the eluant solution, provided an excluding pore size was selected. Any buffer at pH 7.5 or higher could be used as an eluant. Under these conditions, most coat proteins are negatively charged as is the surface of the glass. All chromatographic procedures reported in this paper were performed with sodium tetraborate solution saturated at 4° and containing 1 mM EDTA (pH 9.3). All phages could be stored in this solution without inactivation.

Fig. 1 shows elution patterns of different phages chromatographed on controlled pore glass columns of the pore size that just excluded the respective phage particle. (2250 Å was the largest pore size available, T4 and M13 are certainly excluded by still larger pores.)

The elution patterns of all phages are alike, with the first peak eluted from the column (exclusion volume) containing infective particles and the second peak, well separated from the first, comprising contaminants.

Because the glass has a very narrow pore distribution, M12 and Q β phages could still be excluded by the controlled pore glass with 240-Å pores and Φ X174 was still excluded by 280-Å pores (Fig. 1). These values are very close to the average particle sizes defined by electron microscopy. A high capacity of the columns is reached under these conditions without impairing the resolution of the peaks. Fig. 2 shows an elution profile of Φ X174 raw concentrates purified on a 1 cm $\times$ 100 cm

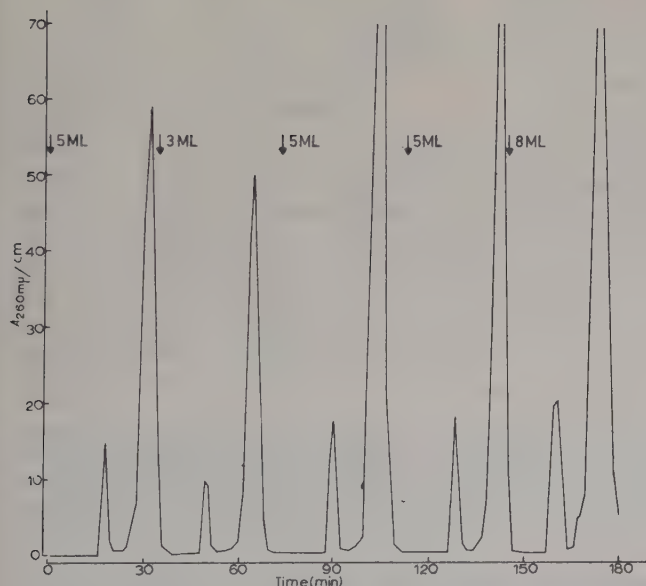


Fig. 2. Purification of Φ X174 phages with repeated sample application. The controlled pore glass column of 280-Å pore size had an exclusion volume of 35.6 ml and a salt volume of 68.2 ml. Flow rate of eluant was 2 ml/min; the times include time of sample injection. The volumes of samples are indicated next to arrows, which mark the positions of sample application. The first peak after each arrow contains the purified phages; the second (larger) peak comprises contaminants.

controlled pore glass column with repeated sample application. This experiment demonstrates that new samples can be applied to the column whenever a salt volume equivalent of buffer from the preceding sample has passed through the column bed. Even after a large series of runs, the base line is not raised. The good reproducibility of the peak positions allows operation of the columns without ultraviolet light monitoring in routine work. The phage-containing fractions are found on the basis of elution volume only. The exclusion volume of the column *minus* half the volume of the sample applied gives the peak position. Flow rates up to 6 ml/min gave good resolution in the most recent experiments, using a fast-working peristaltic pump that produced practically no pulsations. Under these conditions more than 40 ml of raw phage concentrate with titres of $1 \cdot 10^{14}$ infective particles per ml or more could be fractionated in 1 h. This manner of sample processing employed in conjunction with the lysis inhibitory effect of Mg^{2+} (ref. 5) constitutes a fast concentration and purification procedure for $\Phi X174$, M12 and Q β bacteriophages. Some 100 mg of phages can be cultured and purified within 2 days.

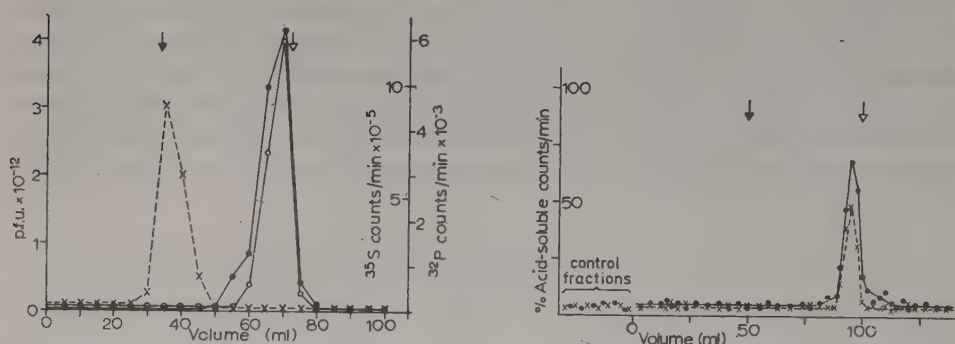


Fig. 3. Elution diagram of radioactively marked host cell protein and nucleic acid components of raw extracts. *E. coli* C bacteria were grown in media that contained ^{35}S or ^{32}P as inorganic sulfate or phosphate from culture densities of $5 \cdot 10^6$ cells per ml to stationary phase. The cells were then washed with an excess of cold sulfate or phosphate to eliminate unincorporated label. $1 \cdot 10^{10}$ cells were suspended in 1 ml of borate-EDTA solution and lysed by lysozyme treatment and by freezing and thawing. The lysates were then mixed with an equal volume of concentrated unlabeled $\Phi X174$ phage lysates, and subjected to the procedure described by GSCHWENDER AND HOFSCHNEIDER⁶. 2 ml of the $10\,000 \times g$ supernatant were chromatographed on a controlled pore glass column of 280-Å pore size. 0.1-ml aliquots of each fraction were dried on slips of filter paper (Whatman III). ^{35}S and ^{32}P activities were determined in a liquid scintillation counter with the paper slips immersed in toluene containing 5 g 2,5-diphenyloxazole and 0.4 g 1,4-bis-(5-phenyloxazolyl-2)benzene scintillation grade. ●—●, ^{35}S activity; ○—○, ^{32}P activity; x---x, infectivity.

Fig. 4. Determination of bacterial nuclease activities after controlled pore glass column chromatography of raw extracts. Log-phase *E. coli* C cells, concentrated to $1 \cdot 10^{10}$ cells per ml and suspended in borate-EDTA solution, were lysed by the procedure described in the legend to Fig. 3. The lysate was ultrasonicated to reduce viscosity and centrifuged for 30 min at $10\,000 \times g$. 2 ml of the supernatant were chromatographed on a controlled pore glass column of 190-Å pore size. 0.5 ml of each fraction was mixed with 1 ml 0.01 M phosphate buffer (pH 7) which contained 0.1 M NaCl, 0.01 M $MgSO_4$ and 3H -labeled *E. coli* DNA (8000 counts/min per ml) and ^{32}P -labeled M12 RNA (20 000 counts/min per ml). The mixture was incubated at 37° overnight. The nucleic acids were precipitated by addition of ice-cold trichloroacetic acid to each fraction to a final concentration of 5 % and collected on membrane filters. The filters were dried and radioactivity was determined as described in the legend to Fig. 3. The percentage of acid-soluble counts was then calculated for each fraction and plotted against volume of eluant. ●—●, % acid-soluble ^{32}P counts representing ribonuclease activity; x---x, % acid-soluble 3H counts representing deoxyribonuclease activity.

As all host bacteria of the bacteriophages dealt with in this paper are *Escherichia coli* strains, there should be no difference in the degree of purity of these phages after controlled pore glass column chromatography. Therefore extensive control experiments to determine purity were made for only one system: the purification of $\Phi X174$ from infected *E. coli* C bacteria. In separate experiments *E. coli* C bacteria were grown in ^{35}S - and in ^{32}P -containing media, concentrated to 1/10 of the culture volume and lysed by lysozyme-EDTA treatment and by freezing and thawing. The lysates were then mixed with an equal volume of unlabeled $\Phi X174$ phage lysate and subsequently treated in the same way as has been described for phage lysates⁵. After chromatography on a 280-Å controlled pore glass column, no radioactivity was found in the exclusion volume in both experiments (Fig. 3). This proves that both protein and nucleic acid components of the host cell, which contaminate raw phage concentrates, are effectively separated from the phage fractions by the chromatographic step.

In additional experiments, the activities of nucleases were tested after controlled pore glass column chromatography because it is especially important to isolate RNA-containing bacteriophages free from ribonuclease. In separate experiments nuclease activities of both bacterial nucleases and the nucleases used for preparing the raw extracts were measured after chromatography of raw extracts on a 190-Å controlled pore glass column (Figs. 4 and 5). No nuclease activity was found in the exclusion volume in both experiments. All nuclease activity is eluted together with the other contaminants. This is confirmed by comparison of the ribonuclease activities in raw phage concentrates and purified fractions shown in Fig. 6.

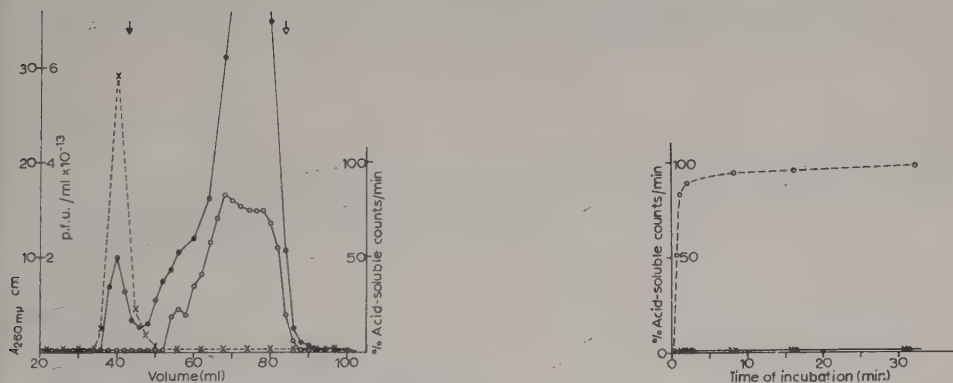


Fig. 5. Ribonuclease activities in an elution diagram of $Q\beta$ phages. 5 ml concentrate containing $Q\beta$ phages, prepared from 1 l of culture as described by Gschwender and Hofschneider⁶, were chromatographed on a controlled pore glass column of 240-Å pore size. Ribonuclease activities were determined in each fraction by the method described in the legend to Fig. 4. ●—●, absorbance at 260 mμ; ○—○, % acid-soluble counts representing ribonuclease activities; ×—×, infectivity.

Fig. 6. Determination of ribonuclease activities in a crude concentrate of M12 bacteriophages ($5 \cdot 10^{13}$ /ml) and the same concentrate after purification by chromatography on a 240-Å controlled pore glass column. 1 ml of sample was mixed with 1 ml 0.1 M phosphate buffer at pH 7.5 containing $5 \cdot 10^6$ counts/min of ^{32}P -labeled M12 RNA and incubated at 37°. At the times indicated 0.1-ml aliquots were withdrawn and pipetted into 5 ml of ice-cold 5% trichloroacetic acid. The percentage of acid-soluble counts was determined as has been described in the legend to Fig. 4. ×—×, control sample with buffer only; ○—○, crude concentrate of M12 bacteriophages; ●—●, the same concentrate after controlled pore glass column chromatography.

These experiments show that controlled pore glass column-chromatographed phage concentrates are purified from all contaminants deriving from the host cell and the culture medium, still present in a 10 000×g supernatant. The purity of controlled pore glass column chromatographed phage fractions is therefore comparable to phage fractions purified by conventional methods, *e.g.* density gradients. The ratios of absorption at 260 m μ to that at 280 m μ were, within experimental error, the same for both controlled pore glass column and density gradient purified phage fractions (Table I). If, however, incomplete phage particles are present in the lysates, *e.g.* 70-S particles in lysates of Φ X174 (ref. 7), these particles are not separated from the infective phages if they have the same size as complete phages. Sedimentation profiles of

TABLE I

RATIOS OF ABSORPTION OF PURIFIED BACTERIOPHAGES

Phage	Ratio of absorption at 260 m μ to 280 m μ		Ref. No.
	After controlled pore glass column chromatography	After density gradient centrifugation	
Φ X174	1.475	1.53	7
M13	1.126	—	
fd	—	1.12*	8
fr	—	1.82*	8
M12	1.78	1.83	9
MS 2	—	1.73*	10
Q β	1.87	1.77*	10

* Values taken from spectra.

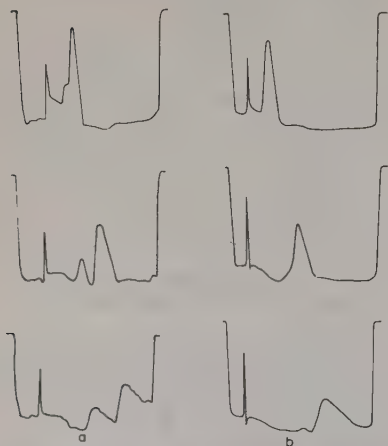


Fig. 7. Typical sedimentation profiles of controlled pore glass column purified bacteriophages. The band-centrifugation technique according to VINOGRAD *et al.*¹¹ was applied. The samples were diluted into 0.1 M NaCl solution to give a concentration of 50 μ g/ml, and 25 μ l were introduced into the pocket of a 30-mm Kel-F centerpiece. The bulk solution was 1 M NaCl. The graph shows densitometer tracings of ultraviolet light photographs taken at 8, 20, and 32 min after the rotor had reached full speed. (a) Φ X174 bacteriophages purified on a 280-Å controlled pore glass column were centrifuged at 44 000×g. (b) Q β bacteriophages purified on a 240-Å controlled pore glass column were centrifuged at 63 000×g.

controlled pore glass column purified $\Phi X174$ and $Q\beta$ phages are shown in Fig. 7. In this case the separation of infective phages and ghosts must be performed by other methods, e.g. density gradient centrifugation, and this can be carried out at great economy after controlled pore glass column chromatography. The phage concentrations can be scaled up and the two particle types are separated in considerably shorter centrifugation times than usual because contaminants have been eliminated by the chromatographic step. For most problems, however, controlled pore glass column purified phages will suffice, e.g. due to the same antigenic structure of $\Phi X174$ and 70-S particles, controlled pore glass purified $\Phi X174$ phages can be used for anti-serum production without further purification. Equally the nucleic acids extracted from controlled pore glass column purified phages could be used as marker substances in density gradients without further purification. Controlled pore glass column chromatography has not only been successfully applied for the quick and economic preparation of large quantities of purified phages, it has also proved to be a very fast and most efficient method for purifying radioactively labeled phages from unincorporated label and all other labeled structures of the host cell.

Finally controlled pore glass column chromatography may also be used for the determination of particle sizes and molecular weights in homologous series of substances, if the unknown particle is purified on a controlled pore glass column of excluding pore size and the peak position of the purified particle is determined on a series of controlled pore glass columns of different pores that admit the particle. From these values the minimal excluding pore size can be extrapolated giving an approximate value for the actual particle size.

In homologous series of substances particle sizes and molecular weights are correlated. Thus, it is also possible to determine molecular weights of particles if peak positions have been determined for other particles of the same group.

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REFERENCES

- 1 W. HALLER, *Nature*, 206 (1965) 693.
- 2 W. HALLER, *J. Chem. Phys.*, 42 (1965) 686.
- 3 W. HALLER, *J. Chromatog.*, 32 (1968) 676.
- 4 W. HALLER, *Virology*, 33 (1967) 740.
- 5 H. H. GSCHWENDER AND P. H. HOFSCHEIDER, *Biochim. Biophys. Acta*, 190 (1969) 454.
- 6 G. HOBOM AND G. BRAUNITZER, *Z. Physiol. Chem.*, 348 (1967) 783.
- 7 R. L. SINSHEIMER, *J. Mol. Biol.*, 1 (1959) 37.
- 8 H. HOFFMANN-BERLING, D. A. MARVIN AND H. DÜRWARD, *Z. Naturforsch.*, 18b (1963) 876.
- 9 H. DELIUS, Thesis, Munich, 1966.
- 10 L. R. OVERBY, G. H. BARLOW, R. H. DOI, M. JACOB AND S. SPIEGELMAN, *J. Bacteriol.*, 91 (1966) 442.
- 11 J. VINOGRAD, R. BRUNER, R. KENT AND J. WEIGLE, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 902.

Lebenslauf

Am 5. April 1943 wurde ich als Sohn des Vermessungsingenieurs Otmar Gschwender und dessen Frau Hilde, geb. Hochreuther, zu Spalt geboren. Meine Kindheit verbrachte ich bei meinen Eltern in Kaufbeuren, wo ich auch die Schule besuchte. Im Juli 1963 legte ich an der Oberrealschule Kaufbeuren die Reifeprüfung ab. Im darauf folgenden Wintersemester 1963/64 begann ich das Studium der Medizin an der Ludwig-Maximilians-Universität zu München. Meine gesamte Studienzeit verbrachte ich in München. Die ärztliche Vorprüfung legte ich im April 1966 ab.

Danach begann ich im Max Planck Institut für Biochemie mit den Experimenten für meine Doktorarbeit.

Im September 1968 heiratete ich meine Studienkollegin Verena Rita Kratzer.

Am 22. 12. 1969 legte ich das ärztliche Staatsexamen ab.

